

**STREPTOMYCES GRISEUS PROTEASE 3 :
PURIFICATION, MECHANISM AND SPECIFICITY.
COMPARISONS WITH SOME OTHER MICROBIAL AND
PANCREATIC SERINE PROTEASES.**

by

Carl-Axel Bauer



Lund 1975

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Purification, mechanism and specificity.
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pancreatic serine proteases**

av

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SUMMARY

An enzyme called Streptomyces griseus Protease 3 (SGP3) has been isolated from the extracellular enzyme mixture Pronase. The purification was accomplished by a single chromatographic step on a specially prepared Sephadex G75 Superfine column. Evidence was obtained for an homogeneous enzyme preparation with an essentially unchanged specific activity.

The catalytic mechanism of the enzyme can be described by the same basic mechanism as for α -chymotrypsin. SGP3 equilibrates rapidly with p-nitrophenylacetate to form a Michaelis complex, followed by a moderately rapid formation of an acyl enzyme and a rate-limiting deacylation of this intermediate.

Both acylation and deacylation of the enzyme is dependent upon an ionizing group with apparent pKa of 6.6, while the Michaelis complex is essentially independent of pH in the region 5.5-10. In the latter respect SGP3 shows similarity with elastase and α -lytic protease, but not with α -chymotrypsin.

SGP3 is irreversibly and equimolarly inhibited by diisopropylfluorophosphate, thus permitting its classification as a serine enzyme. In contrast to α -chymotrypsin, the enzyme is only partly inhibited by tosyl phenylalanine chloromethyl ketone.

Like α -chymotrypsin, SGP3 has a preference for the acyl bonds of tyrosine, phenylalanine and leucine. However, SGP3 hydrolyzes bonds C-terminal to short amino acid residues more rapidly than does α -chymotrypsin. The active site of SGP3 extends over about 7 subsites and that of α -chymotrypsin over about 6. There appear to be important differences in the functions of these active sites since the hydrolysis rate of SGP3 is very sensitive to peptide chain length, whereas α -chymotrypsin is not. α -Chymotrypsin, but not SGP3, rapidly hydrolyses specific acetyl amino acid amides, and this enzyme depends mainly on primary enzyme-substrate contacts. SGP3, in contrast, depends mainly on secondary enzyme-substrate contacts with amino acid residues more remote from the scissile bond, in this respect showing close similarity with elastase.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I. Bauer, C.-A., and Löfqvist, B. (1973): "Studies of the Heterogeneity of Streptomyces griseus Protease. Isolation and Characterization of an Alkaline Serine Protease from Commercial Pronase-P Derived from Streptomyces griseus K 1. Acta Chem. Scand. 27, 3147-3166.
- II. Bauer, C.-A., Löfqvist, B., and Pettersson, G. (1974): "Studies on the Catalytic Mechanism of a Serine Protease from Streptomyces griseus". Eur. J. Biochem. 41, 45-49.
- III. Bauer, C.-A., and Pettersson, G. (1974): "Effect of pH on the Catalytic Activity of Streptomyces griseus Protease 3". Eur. J. Biochem. 45, 469-472.
- IV. Bauer, C.-A., Thompson, R.C., and Blout, E.R.: "The Active Centers of Streptomyces griseus Protease 3 and α -Chymotrypsin: Enzyme-Substrate Interactions Remote from the Scissile Bond". Submitted for publication.
- V. Bauer, C.-A., Thompson, R.C., and Blout, E.R.: "The Active Centers of Streptomyces griseus Protease 3, α -Chymotrypsin, and Elastase: Enzyme-Substrate Interactions Close to the Scissile Bond". Submitted for publication.
- VI. Bauer, C.-A.: "The Active Centers of Streptomyces griseus Protease 3 and α -Chymotrypsin: Enzyme-Substrate Interactions Beyond Subsite S₁". In manus.

ABBREVIATIONS

Ac- = N-acetyl

Amino acids have been abbreviated according to accepted nomenclature . All amino acid residues mentioned in this thesis are of the L-configuration, unless otherwise stated.

-NH₂ = amide

S_x-P_x. The nomenclature introduced by Schechter and Berger (1967) is used to facilitate discussion of the interactions between a protease and bound peptides. Amino acid residues and partial amino acid residues (e.g., acetyl groups) of substrates are numbered P₁, P₂, P₃, etc., in the N-terminal direction, and P₁['], P₂['], etc., in the C-terminal direction from the scissile bond. The complementary subsites of the enzyme's active center are numbered S₁, S₂ and S₁['], S₂['], etc., in an analogous fashion. The binding mode of a peptide which occupies, for example, the S₄, S₃, S₂, and S₁ subsites of the enzyme will be denoted by the abbreviation S₄₃₂₁.

SGP3 = Streptomyces griseus Protease 3 has been designated number 3 because of its migration position in the high resolution gel electrophoretic system of Löfqvist and Sjöberg (1971). Since this system presently gives the best resolution of the Pronase complex, it provides a rapid and efficient system for identifying Pronase components from different laboratories.

SGP3 has been shown (Löfqvist and Sjöberg, 1971; Bauer and Löfqvist, 1973) to be identical to "PNPA-hydrolase I", described by Wählby (1968), "alkaline protease a" (Narahashi, 1970), "Streptomyces griseus Enzyme II" (Gertler and Trop, 1971), "Streptomyces griseus protease A" (Johnson and Smillie, 1971), and "lysine-free chymoelastase" (Siegel and Awad, 1973).

INTRODUCTION

During production of the antibiotic streptomycin by the microorganism Streptomyces griseus (strain K1) it was found that a remarkable amount of protease was excreted into the culture broth (Nomoto and Narahashi, 1959 a). The isolated protease, called Pronase, was found to exhibit an unusually broad specificity (Nomoto et al., 1960 a, b), despite its characterization as an homogeneous enzyme (Nomoto and Narahashi, 1959 b). Later work showed, however, that Pronase is a complex mixture of many proteolytic enzymes. For example, Narahashi et al. (1968) gave evidence for the presence of 11 different, enzymatically active components, and, by gel electrophoresis, Löfqvist and Sjöberg (1971) obtained evidence for 13 active components. It is now well established that Pronase contains at least 4 serine proteases (Gertler and Trop, 1971; Awad et al., 1972; Löfqvist, 1974 b), 2 or more neutral proteases (Narahashi et al., 1968; Löfqvist, 1974 a), 1 carboxypeptidase (Narahashi et al., 1968; Löfqvist, 1974 a) and at least 2 aminopeptidases (Narahashi et al., 1968; Vosbeck et al., 1973; Löfqvist, 1974 a). Several of these enzymes have been isolated on a preparative scale, the best characterized group being the serine proteases. One of the serine proteases appears to be subtilisin-like (Awad et al., 1972), while the other three belong to the chymotrypsin-family of proteases (Wählby and Engström, 1968; Johnson and Smillie, 1971; Awad et al., 1972). Of the latter three, one is a trypsin-like enzyme (Trop and Birk, 1968; Olafson and Smillie, 1975; Olafson et al., 1975), while the two others have been characterized as chymotrypsin-elastase like (Gertler and Trop, 1971; Johnson and Smillie, 1971; Narahashi and Yoda, 1973; I).

The presence of so many different proteases in Pronase has caused great interest, both from practical and theoretical points of view. For instance, the use of such a potent mixture of proteases is helpful both in industrial protein chemistry and for the preparation of protein free material in the laboratory. Apart from the fact that the Pronase complex, in itself, poses interesting questions as to heterogeneity, specificity and stability, several of the Pronase components appear to be of interest both from evolutionary, functional and genetic points of view.

For many years the Pronase complex has been, and still is, a challenge for protein purification methods. Obviously, the many different proteases in Pronase mean that special care must be taken during purification procedures, because of the risk for total or limited proteolysis of some, or all, of them. The use of

ion-exchange chromatography, for example, may sometimes be hazardous since non-optimal pH:s and media may have to be used. The development of a highly efficient gel chromatography method, using fractionated Sephadex G75 Superfine, has provided a one-step purification method for certain of the Pronase components, under optimal conditions of stability (Löfqvist and Klevhag, 1974). One of these components is a serine enzyme, called Streptomyces griseus Protease 3 (SGP3), and is the subject of the present studies.

Initially, the aim of studying SGP3 was to achieve a better understanding, in connection with studies on other Pronase components, of such properties of Pronase as specificity, stability and pH and temperature optima. However, being a member of the chymotrypsin-family of proteases, SGP3 proved to be an interesting tool for comparing certain properties of microbial and mammalian pancreatic serine proteases. Detailed sequence (Johnson and Smillie, 1974), mechanism (II-III) and specificity studies (I, IV-VI) of SGP3, in relation to some other proteolytic enzymes, have now been reported.

A summarized discussion of purification procedures, mechanism, pH-dependence, inhibitors and specificity, with emphasis on comparisons with some other microbial and pancreatic serine proteases, is presented on the following pages. Although no studies on the structure of SGP3 have been made by the author, this is considered of such general importance for the understanding of relations between SGP3 and other proteases, that the current knowledge of the structure of SGP3 is briefly reviewed.

PURIFICATION

Purification to homogeneity of SGP3 was first reported by Wählby (1969), the purification method including several different chromatographic steps on ion-exchangers and Sephadex G50. Quite a poor yield of SGP3 was obtained, but gel electrophoretic evidence indicated that the identity of the enzyme had not been altered during the purification procedures. Subsequently, similar purification methods for SGP3 were reported by other investigators (Narahashi and Fukunaga, 1969; Gertler and Trop, 1971; Awad et al., 1972). The purification method described by Johnson and Smillie (1971) is especially noteworthy. This method included lengthy ion-exchange chromatography and dialysis-periods, and yielded two forms of SGP3, having the same amino acid contents, but different specific activities. The heterogeneity was thought to be due to limited autolysis during the isolation procedures.

A one-step purification method for SGP3 was described by Jurasek et al. (1971). However, prior to the ion-exchange step, Pronase was dialyzed at pH 5.0 overnight, under which conditions several of the Pronase components were totally digested (Löfqvist and Klevhag, 1974). It appears, however, that most of the SGP3 was recovered.

Clearly, poor yield and the risk of limited autolysis of SGP3 during dialysis and ion-exchange chromatography, call for methods permitting the purification to be achieved under optimal conditions of stability. Such a purification method based on gel chromatography, has been developed by Löfqvist and Klevhag (1974), the main feature of this procedure being the use of fractionated Sephadex G75 Superfine. With this method, Pronase can be applied to the chromatographic column immediately after solubilization and chromatography can be accomplished under optimal conditions of stability.

In publication I, special attention has been directed towards the abovementioned purification problems and an attempt to check both yield and specific activity of SGP3 during the purification has been made. The results indicate that the specific activity of the purified enzyme has not been markedly altered. Further support for the homogeneity of the preparation is indicated by the appearance of only one band in gel electrophoresis. Earlier microheterogeneities have been thought to be due to deamidation (Johnson and Smillie, 1971), and such enzyme forms, which have different charges would not be expected to migrate together in the gel electrophoresis method used. The high yield, furthermore, is a good indication that optimal stability conditions have been achieved during this separation.

It is evident from I: Fig. 1 that there is a remarkable resolution of the Pronase components on the 10 x 90 cm Sephadex G75 Superfine column. The factors contributing to this high resolution have been discussed by Löfqvist and Klevhag (1974). It may be worthwhile to point out, however, that the most important factors for the resolution are

- a) the use of fractionated Sephadex G75 Superfine with particles $> 40 \mu$, which greatly improves flow properties. Moreover, there is evidence that such particles have a more homogeneous pore size (Löfqvist, unpublished).
- b) care being taken not to overload the column by applying too high amounts of protein, which would reduce the resolution.

- c) special care being taken on the application of the Pronase solution, to avoid tailing of the peaks.

Nevertheless, it is surprising that it is possible to achieve a complete separation between SGP3 (peak G) and F (main component SGP1), since the molecular weights for these enzymes, from sequential analysis, have been determined to 18,064 and 18,412, respectively (Jurasek et al., 1974). By gel filtration the molecular weights have been estimated at 15,500 (SGP3) and 17,500 (Awad et al., 1972) and at 15,000 (SGP3) and 18,000 (Löfqvist, 1974 b). Since the estimations for SGP3 are appreciably lower than the molecular weight obtained from sequential analysis, it is possible that there may be some kind of special interaction between Sephadex and SGP3, slightly retarding the enzyme.

Finally, it may be pointed out that the combination of Sephadex gel chromatography and hydrophobic affinity chromatography may prove to be useful for further increasing the resolution of the remaining, unresolved Pronase components, under optimal conditions of stability. The combination of these two chromatography methods may, of course, also be a possible means of purifying SGP3 if a less sophisticated Sephadex column is used.

STRUCTURE

The first structural studies on SGP3 were reported by Wählby and Engström (1968), who showed the amino-acid sequence around the reactive seryl residue to be Asp-Ser-Gly. Later, Awad et al. (1972) confirmed this data, while Johnson and Smillie (1971) made an extensive study both of the sequence around this seryl residue and around the two disulphide bridges. The latter study gave further evidence for the relationship between SGP3 and the mammalian pancreatic serine proteases.

Recently, the complete amino-acid sequence and predicted structure of SGP3 were published (Johnson and Smillie, 1974). It was shown that SGP3 is a single polypeptide chain of 182 amino-acid residues with two disulphide bridges. Comparison of the primary structure of SGP3 with those of two other microbial serine proteases, SGP1 and Myxobacter α -lytic protease, shows that the identity of residues between SGP3 and SGP1 is 61% (Jurasek et al., 1974) and between SGP3 and α -lytic protease 31%. The identity of residues between SGP3 and porcine elastase and bovine chymotrypsinogen A is only 11 and 12%, respectively (Johnson and Smillie, 1974). However, for all 5 enzymes there is a remarkable

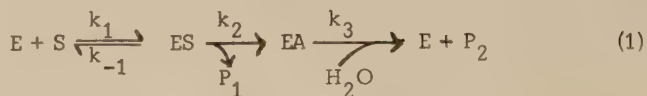
sequence identity around the catalytic triad Asp 102, His 57 and Ser 195 and around the two disulphide bridges (Cys 42 to Cys 58 and Cys 191 to Cys 220). It is predicted, therefore, that the tertiary structure in these regions will also be very similar. The overall tertiary structure of the microbial enzymes are thought to differ from the mammalian mainly in three surface regions where the former enzymes possess alterations in the sequences (Johnson and Smillie, 1974).

Because SGP1, in contrast to SGP3, is remarkably resistant to denaturation in 6 M guanidine hydrochloride (Siegel et al., 1972) it is of great interest to establish which factors in the enzyme architecture are important for this stability. The amino acid sequence of the two enzymes are, however, very similar, except between residues 60 and 88 G and between 108 and 135 (Jurasek et al., 1974). Before any firm conclusions can be drawn as to the factors contributing to the stability of SGP1, it is clear that detailed X-ray structures of both enzymes must be determined.

The finding that SGP3 belongs to the chymotrypsin-family of proteases, puts it apart from the subtilisins, a group of exclusively bacterial serine proteases. There is no similarity either in the primary structures of subtilisin BPN and α -chymotrypsin (Smith et al., 1966) or in their three-dimensional structures (Wright et al., 1969). Interestingly, however, both enzymes possess the same catalytic triad Asp, His, Ser and it appears that the topology of the hydrogen bond networks are similar in the triads (Kraut, 1971). It is thought, therefore, that these two families of serine proteases represent a case of independant convergent evolution at the molecular level (Smith et al., 1966; Kraut, 1971).

CATALYTIC MECHANISM

α -Chymotrypsin-catalyzed ester hydrolysis has been found to conform basically to the minimal kinetic scheme



where E is free enzyme, ES Michaelis complex, EA acyl enzyme, S substrate and P_1 and P_2 reaction products (Gutfreund and Sturtevant, 1956; Kézdy and Bender, 1962; Bender and Kézdy, 1965). Studies of two other pancreatic ser

proteases, trypsin and elastase, gave evidence that their mechanisms, in principal, were consistent with this scheme (Bender and Kézdy, 1965; Bender and Marshall, 1968). Interestingly, the bacterial serine enzyme α -lytic protease, which shows considerable structural homologies with the pancreatic serine proteases around the catalytic residues, was also found to catalyze the hydrolysis of p-nitrophenyl acetate and p-nitrophenyl trimethylacetate by a mechanism consistent with scheme (1) (Kaplan and Whitaker, 1969). The finding of a common basic reaction mechanism for these enzymes was of considerable importance, since it had earlier been thought that two histidyl residues were essential for catalysis by pancreatic proteases (Bender and Kézdy, 1964) whereas α -lytic protease contains only one histidyl residue (Kaplan and Whitaker, 1969). This finding emphasizes the importance of homology studies.

Studies on the catalytic mechanism of SGP3 with p-nitrophenyl acetate gave evidence for a mechanism consistent with scheme (1) (II). This provided further evidence that, despite the widely differing phylogenetic origin of the enzymes, the catalytic mechanism of these bacterial and mammalian pancreatic serine proteases can be expressed in a common scheme.

Studies of α -chymotrypsin-catalyzed hydrolysis of amides, anilides and peptides have confirmed that these reactions, also, can be described by the minimal kinetic scheme (1) (Berezin et al., 1973; Fastrez and Fersht, 1973). Although there is no direct proof that hydrolysis of amides, anilides and peptides, catalyzed by elastase and the bacterial serine enzymes, proceeds by the same mechanism as for α -chymotrypsin, this has been accepted as a highly probable hypothesis in view of the common mechanism for ester hydrolysis and the striking sequential similarities between the enzymes. However, although the catalytic mechanism in principal can be expressed in a common scheme, it is not clear, a priori, whether or not the rate-determining steps for ester or "peptide type" hydrolysis are the same for the different serine proteases. For SGP3 the indications are that, as in α -chymotrypsin, k_2 is the rate-determining step in the hydrolysis of anilides, amides and peptides (III, VI). For certain methyl esters, however, the rate-determining step of the reaction appears to differ for the two enzymes (Bauer, unpublished) and this phenomenon is now under investigation.

A more detailed discussion of catalysis would include such features as transition state intermediates and factors which may contribute to catalysis. However, such factors are considered to be beyond the scope of the present work and are not discussed in this thesis.

pH-DEPENDENCE

Chymotrypsin-catalyzed reactions exhibit bell shaped pH profiles with an optimum around pH 8.0 for the hydrolysis of a neutral substrate. Such pH profiles are often interpreted as indicating the presence of two catalytically important groups in the enzyme, the pKa of these groups being about 7 and 8.5 for α -chymotrypsin (Hess, 1971). There is considerable evidence that the ionizing group, with a pKa of about 7, is the imidazole group of His 57 of the active site, whereas the group with a pKa of about 8.5 is thought to be the N-terminal amino acid Ile 16 (Hess, 1971). Blow et al. (1969) have postulated that His 57 is part of a so called "charge-relay" system at the active site of α -chymotrypsin, consisting of Asp 102, His 57 and Ser 195. It has been suggested that the negatively charged carboxyl group of Asp 102 can transfer electrons through hydrogen bonds and the polarizable imidazole of His 57 to the Ser 195 oxygen, thus making that oxygen more nucleophilic (Blow et al., 1969). X-ray crystallographic studies of pancreatic elastase, an enzyme homologous to chymotrypsin, have shown that a "charge-relay" system similar to that of α -chymotrypsin, also exists in elastase (Hartley and Shotton, 1971).

SGP3 is also a member of the chymotrypsin-family of proteases (Johnson and Smillie, 1971), and it could, therefore, be expected to have a similar "charge-relay" system. A preliminary study of the pH-dependence of SGP3 did not give any evidence for a pKa below 9 (I), thus indicating important mechanistical differences between SGP3 and the pancreatic serine proteases. However, since borate buffer had been used in that study and since there is some evidence that boric acid functions as an inhibitor of α -chymotrypsin (Berezin et al., 1966), it could not be excluded that the reported abnormal pH-dependence of SGP3 was due to interactions between the enzyme and the borate buffer. Therefore, a more detailed study of the pH-dependence of SGP3 was carried out, using buffers other than borate (I) (III). This investigation provided evidence that both acylation and deacylation of the enzyme are dependent on an ionizing group in the protein with $pK_a \approx 6.6$, which is in good agreement with corresponding values reported for α -chymotrypsin (Hess, 1971) and elastase (Hartley and Shotton, 1971). However, unlike α -chymotrypsin, but like elastase (Hartley and Shotton, 1971) and α -lytic protease (Kaplan and Whitaker, 1969), K_m for SGP3 is essentially constant between pH 5.5 and 10, meaning that k_{cat}/K_m is independent of pH between 8 and 10 (III).

The rapid decrease in chymotrypsin activity above pH 8 has been related to an ion pair bond between residues Asp 194 and Ile 16 (Hess, 1971). Because the apparent pKa is approximately 8.5 for the N-terminal Ile 16, the ion pair bond will be considerably weakened above pH 8 which is thought to result in a conformational change in the enzyme causing decreased ability to bind the substrate (Hess, 1971). As mentioned above, this situation is different in elastase, α -lytic protease and SGP3, since in all three enzymes, k_{cat} and K_m are independent of pH between at least pH 8 and 10. Like α -chymotrypsin SGP3 has a N-terminal Ile 16, while elastase has a Val, and all the enzymes possess an Asp 194 (Johnson and Smillie, 1974). X-ray crystallographic studies of elastase have shown that there is an ion pair bond between Val 16 and Asp 194, the apparent pKa of Val 16 being estimated at 9.7 (Hartley and Shotton, 1971). However, the internal ion pair of Val 16 and Asp 194 does not seem to be essential for the activity of elastase since the kinetic constants are unchanged around pH 10. Furthermore, acetylation of the aminogroup of Val 16, thereby preventing it becoming positively charged and forming an ion pair bond, does not affect the catalytic activity of the enzyme (Hartley and Shotton, 1971). N-acetylation of SGP3 did not cause any appreciable change in K_m for this enzyme either (Siegel and Awad, 1973), and it was suggested that the ion-pair bond between N-terminal Ile/Val and Asp 194 is simply replaced by a hydrogen bond upon acetylation, explaining the retention of activity in SGP3 and elastase. However, this does not explain the differences between α -chymotrypsin on the one hand and elastase, SGP3 and α -lytic protease on the other. It is clear that more knowledge about the structure of the enzymes is needed before the factors behind the different pH-optima is understood at the molecular level.

Another point concerning the pH-dependence of the serine proteases, which recently has aroused much interest, is the group responsible for the apparent pKa of about 7. As mentioned above, this group has long been thought to be the imidazole group of His 57. However, the single histidine residue of α -lytic protease has been selectively enriched in the C-2 carbon of the imidazole group with ^{13}C , and ^{13}C n.m.r. studies have been interpreted as showing that the ionizing group with a pKa around 7 is Asp 102 and not His 57 (Hunkapiller et al., 1973). This interpretation has been questioned by Robillard and Schulman (1974) who stated that ^1H n.m.r. studies were consistent with His 57 being the ionizing group. To further complicate the situation, it has recently been questioned whether the "charge-relay" system is really a "charge-relay" system (Brinigar and Chao, 1975) (!).

It is evident, then, that although the α -chymotrypsin-family of enzymes constitute one of the best, if not the best, understood group of enzymes, several important points, exemplified above, still await definitive answers.

INHIBITORS

The use of inhibitors which react specifically with amino acid residues or cofactors important for the enzymatic catalysis, is often very helpful for the classification of enzymes and can give important clues to the catalytic mechanism. For the proteases, ethylenediaminetetraacetate, diisopropylfluorophosphate and p-chloromercuribenzoate have proved to be specially important for primary classification purposes.

SGP3 is rapidly and equimolarly inactivated by diisopropylfluorophosphate, which reacts with a specific seryl residue thought to be at the active site (Wählby and Engström, 1968; Wählby 1969), thus permitting the classification of SGP3 as a serine enzyme (i.e. an enzyme with a specially reactive seryl residue involved in catalysis). The reactivity of SGP3 towards diisopropylfluorophosphate has been confirmed by all subsequent investigators.

Another group of inhibitors which permit further classification of the structure of the active site of proteases are the chloromethyl ketones. For example, the chloromethyl ketones of tosyl phenylalanine and tosyl lysine have been found to react irreversibly with His 57 at the active sites of chymotrypsin and trypsin, respectively (Schoellman and Shaw, 1963; Shaw et al., 1965). However, attempts to inhibit SGP3 with tosyl phenylalanine chloromethyl ketone in order to obtain evidence of similarity with α -chymotrypsin and for the presence of an active site histidyl residue yielded only a partly inhibited enzyme (Gertler and Trop, 1971; Johnson and Smillie, 1971; I). Chloromethyl ketones of some other tosylamino acids were even less effective than the tosyl phenylalanine analogue (I). Peptide chloromethyl ketones, however, have been found to react more rapidly than tosylamino acid chloromethyl ketones with chymotrypsin (Kurachi et al., 1973) and subtilisin (Moriwara and Oka, 1970). Moreover, Thompson and Blout (1973 a) have shown that the rate of inactivation of elastase by peptide chloromethyl ketones increased dramatically with increasing peptide chain length. A parallel dependence of the ease of substrate hydrolysis on peptide chain length was observed for that enzyme (Thompson and Blout, 1973 c). The fact that a similar chain-length dependence is observed in SGP3 (IV), indicates that peptide chloromethyl ketone analogues of good SGP3 substrates, like Ac-Pro-Ala-Pro-Phe-NH₂,

may be efficient irreversible inhibitors and explains the poor reactivity of SGP3 towards tosylamino acid chloromethyl ketones. The syntheses of potentially suitable peptide chloromethyl ketones are now under way in the author's laboratory. Recently, it has indeed been shown that SGP1, being closely related to SGP3, reacts much more rapidly with chloromethyl ketones with extended peptide chains than with shorter ones (Gertler, 1974).

Boric acid and boronic acid derivatives have aroused much interest since they act as reversible inhibitors for chymotrypsin and subtilisin (Berezin et al., 1966; Philipp and Bender, 1971). While it was initially believed that these inhibitors bind to the active histidyl residue (Berezin et al., 1967; Philipp and Bender, 1971), there is now evidence that they bind to the active seryl residue, forming a tetrahedral adduct, thought to be of certain similarity to the transition state complex (Koehler and Lienhard, 1971; Lindquist and Terry, 1974). This has recently been confirmed by crystallographic studies of complexes between certain boronic acids and subtilisin BPN (Birktoft and Matthews quoted in Lindquist and Terry, 1974). It has been found that boric acid also binds to SGP3 and some evidence has been presented, indicating that boric acid interacts with SGP3 in a way analogous to the abovementioned proteases (Bauer and Pettersson, 1974).

A new class of reversible enzyme inhibitors of particular interest for the study of proteases is the peptide aldehydes. Such inhibitors have been found to bind very tightly to elastase (Thompson, 1973) and papain (Westerik and Wolfenden, 1972), their K_i being lower, by several orders of magnitude than corresponding analogues such as peptide alcohols and amides. In addition, aldehydes prefer to exist as tetrahedral addition complexes, and it is therefore believed that peptide aldehydes are good transition state analogues for those proteases which form tetrahedral intermediates with their substrates during catalysis. Very recently a couple of peptide aldehydes have been synthesized and tested on SGP3 and α -chymotrypsin (Bauer and Thompson, in prep.). The best inhibitor, Ac-Pro-Ala-Pro-Phe-aldehyde, has $K_i = 5 \times 10^{-8} \text{ M}$ for SGP3 and $K_i = 3 \times 10^{-6} \text{ M}$ for α -chymotrypsin, thus binding about 10^4 and 10^3 times more strongly, respectively, than corresponding alcohols and amides.

SPECIFICITY

The first specificity studies of SGP3 were reported by Gertler and Trop (1971) and Johnson and Smillie (1971). Gertler and Trop (1971) found that SGP3 possessed strong esterolytic activity towards the specific elastase substrate Ac-Ala₃ methyl ester as well as high activity towards congo red elastin, while the activity towards Ac-Tyr ethyl ester, a specific chymotrypsin substrate, was lower. Johnson and Smillie (1971) investigated the specificity of SGP3 towards the oxidized insulin A and B chains, and found that the enzyme had a wide specificity, hydrolyzing the peptide bonds C-terminal to aromatic and aliphatic amino acid residues, and, more surprising, also peptide bonds C-terminal to Arg, Lys, and Glu. Later studies on the specificity of SGP3, using small synthetic substrates, showed that it was directed towards aromatic and aliphatic amino acid side chains, and SGP3 was therefore considered to be "chymotrypsin elastase like" (I; Trop, 1973).

The kinetic constants for the esterase activity of SGP3 on going from Ac-Ala methyl ester to Ac-Ala₃ methyl ester indicated the existence of an extended active site in SGP3 (I). Later studies have shown that the active site of SGP3 extends over several subsites and that these, furthermore, are of great importance in increasing the rate of hydrolysis (IV - VI). The specificity of SGP3 will, therefore, be discussed in terms of a primary specificity, manifested in S₁-P₁ interactions, and a secondary specificity, manifested in enzyme-substrate interactions outside the S₁ subsite. Finally, an attempt is made to discuss the relationship between these two kinds of specificity, with special attention to the structure of the S₁ subsite.

Primary specificity.

A preliminary investigation of the S₁ specificity was conducted with a series of carbobenzoxy amino acid p-nitrophenylesters and clearly indicated a close similarity with α -chymotrypsin (I). However, because K_m for this kind of substrate is not easily correlated with K_s and, moreover, because p-nitrophenyl esters are comparatively nonspecific substrates (Silver and Matta, 1972), it has been desirable to further compare the specificity of the S₁ subsites of SGP3 and α -chymotrypsin.

A series of tetrapeptide amides has been prepared, which appear to bind to α -chymotrypsin and SGP3 in only one way, thus permitting the primary specificity of the enzymes to be assessed in terms of binding energies and acylation

rates (IV, V). Clear evidence was obtained that the S_1 subsite of SGP3, like that of α -chymotrypsin, shows a preference for aromatic and long chain aliphatic amino acids (V). However, SGP3 has a higher activity and binds aliphatic amino acid residues more strongly than α -chymotrypsin. On the other hand, a comparison between the activity of SGP3 and elastase towards Ac-Pro-Ala-Pro-Phe-NH₂, an excellent elastase substrate (Thompson and Blout, 1973 c), shows that SGP3 hydrolyzes that substrate at only about 2% of the rate of elastase.

Johnson and Smillie (1971) reported that SGP3 also hydrolyzed peptide bonds C-terminal to arginyl residues in insulin to a significant degree. However, the preparation of SGP3 obtained by the method described in paper I, shows very little activity towards benzoyl Arg methyl ester and no activity at all towards benzoyl D,L-Arg p-nitroanilide (I). Independently, Trop (1973) reported similar results for another preparation of SGP3, thus further strengthening the view that the S_1 subsite of SGP3, in itself, does not have any preference for arginyl residues.

From the data presented in papers I and V, it is evident that the specificity of the S_1 subsite of SGP3 has much in common with that of α -chymotrypsin. A very important difference between SGP3 and α -chymotrypsin, however, is found in the ability to hydrolyze Ac-Phe-NH₂, for which substrate k_{cat} is about 30 times lower for SGP3 than for α -chymotrypsin (IV). The reason for this difference between the two enzymes will be discussed in the following sections.

Secondary specificity.

The so called secondary specificity of enzymes is manifested in enzyme-substrate interactions outside the S_1 subsite and has been shown to exist in a number of enzymes which modify macromolecules. Among the proteases, there is evidence that the active sites of papain (Schechter and Berger, 1967) and elastase (Atlas et al., 1970) extend over about 7 subsites. The data in papers IV and VI indicate that the active site of SGP3 extends over about 7 subsites, also, and that of α -chymotrypsin over about 6 subsites. The importance of such extended active sites for substrate binding and acylation rate is more difficult to assess, since that requires substrates which bind in one principal mode, and for which K_m can easily be equated with K_s and k_{cat} with k_2 or k_3 .

In a series of elegant papers, Thompson and Blout (1970; 1973, b-d) showed that elastase is extremely dependent on peptide chain length N-terminal to the scissile bond, k_{cat}/K_m increasing more than 4×10^5 -fold on going from Ac-Ala-NH₂ to Ac-Pro-Ala-Pro-Ala-NH₂. The use of proline-containing peptides, which were shown to bind in one principal mode, only (Thompson and Blout, 1973 b), permitted evaluation of the relative importance of binding and acylation rate in the dramatic increase of k_{cat}/K_m . By far the most important factor was found to be the acylation rate, which is extremely dependent on substrate chain length (Thompson and Blout, 1973 c). In contrast, the deacylation rate is not very sensitive to substrate chain length (Thompson and Blout, 1970).

For α -chymotrypsin, there is evidence that enzyme-substrate interactions N-terminal to the scissile bond can increase k_{cat}/K_m about 100-fold, on going from Ac-Phe-NH₂ to Ac-Pro-Ala-Pro-Phe-NH₂ (IV). This is due to similar increases in both the binding and the acylation rate. Essentially similar results have been found for α -chymotrypsin-catalyzed hydrolysis of peptides (Baumann et al., 1973). There is also evidence that enzyme-substrate interactions C-terminal to the scissile bond at subsite S₁' and, to a smaller extent, at subsite S₂' are of considerable importance in the rate of hydrolysis in α -chymotrypsin (V, VI).

The activity of SGP3 has been tested towards the same set of amides as used for α -chymotrypsin (IV). It was found that SGP3 is much more sensitive to peptide chain length than α -chymotrypsin, k_{cat}/K_m increasing about 4×10^4 -fold on going from Ac-Phe-NH₂ to Ac-Pro-Ala-Pro-Phe-NH₂. Since it appears that the proline-containing phenylalaninamide peptides also bind in only one principal mode, it has been possible to show that, unlike α -chymotrypsin, the acylation rate of SGP3 is highly sensitive to substrate chain length, increasing about 800-fold in the above mentioned series (IV). SGP3 is also considerably more sensitive to enzyme-substrate interactions C-terminal to the scissile bond than α -chymotrypsin (V, VI).

The relationships between primary and secondary specificity.

Of the three enzymes α -chymotrypsin, elastase and SGP3, the only one which appears to be reasonably effective in hydrolyzing substrates with specificity determinants present only in P₁ and the proximal part of P₂ is α -chymotrypsin (IV). However, when elastase and SGP3 are able to form contacts with their substrates outside the "primary specificity site", their ability to hydrolyze is greatly increased (IV). It appears that formation of the S₄₃₂-P₄₃₂ contact in

SGP3 (IV) and the $S_{54}-P_{54}$ contact in elastase (Thompson and Blout, 1973 c) are especially important in increasing the acylation rate. According to Thompson and Blout (1973 c), this means that the $S_{432}-P_{432}$ and $S_{54}-P_{54}$ enzyme-substrate contacts are capable of correctly orienting the P_1-P_1' bond with respect to S_1 and S_1' , and that there must be an information transfer from these remote enzyme-substrate contacts to the $S_{11'}-P_{11'}$ contacts. Such information could be transmitted either along the substrate peptide chain or through the enzyme structure. For elastase there is now considerable evidence that information, mediated through the enzyme structure, induces a rearrangement of the S_1 and S_1' subsites (Thompson and Blout, 1973 c; Thompson, 1974). Although there is no evidence as to how information transfer occurs in a complex between SGP3 and a substrate, it would seem a reasonable guess that it is transmitted in a way similar to elastase, in view of the structural similarities between the enzymes.

X-ray crystallographic studies of α -chymotrypsin-substrate complexes have shown that aromatic and long-chain aliphatic amino acid residues fit into a deep pocket in the enzyme (Steitz et al., 1969). It appears that the fit of this pocket and its ability to restrict the motion of the C^α in the enzyme-substrate complex are important factors in the comparatively high acitivity of α -chymotrypsin towards short substrates like Ac-Phe-NH₂.

The specificity of elastase is directed towards short amino acid side chains, primarily alanyl residues (Kaplan et al., 1970; Thompson and Blout, 1973 c). This is consistent with the X-ray structure of elastase which revealed that the mouth of the binding pocket is closed by the side chains of Val 216 and Thr 226, thus excluding larger amino acid side chains (Hartley and Shotton, 1971). It is possible, therefore, that the methyl side chain of Ala will be much less strongly bound and much less restricted in its motions than a benzylgroup bound to the pocket in α -chymotrypsin. Apparently, elastase is able to overcome the resulting poor orientation of the P_1 group when additional enzyme-substrate contacts are being formed outside the primary specificity site.

In SGP3 we observe the same binding energy for a benzylgroup as in α -chymotrypsin (V). However, SGP3 splits Ac-Phe-NH₂ about 30 times slower than α -chymotrypsin (IV). It can be expected, therefore, that the S_1 binding site in SGP3 is not able to restrict the motion of the benzyl group as effectively as in α -chymotrypsin or, alternatively, that the catalytic groups, in themselves, are not optimally oriented with respect to the scissile bond. As in elastase,

this inability is overcome when enzyme-substrate contacts outside the S_1 subsite can be formed (IV). Thus, although SGP3 is a member of the chymotrypsin-family of proteases and although SGP3 shows similar binding ability and size of the active site as α -chymotrypsin it is evident that there are fundamental differences in the functions of the active sites of α -chymotrypsin and SGP3. In this respect it appears that SGP3 has much more in common with elastase. In this context, it appears that a detailed study of the primary and secondary specificities of α -lytic protease and SGP1 would provide a most interesting comparison.

There may also be several similarities between SGP3 and subtilisin BPN, a bacterial serine enzyme not belonging to the chymotrypsin-family of enzymes. Subtilisin BPN exhibits a broad substrate specificity, primarily hydrolyzing bonds C-terminal to aromatic and aliphatic amino acid residues (Mori-hara and Tsuzuki, 1969). Although the secondary specificity of subtilisin BPN has not been studied in detail, it appears that the enzyme is highly dependent on substrate chain length (Mori-hara and Oka, 1973 a). Detailed X-ray crystallographic studies have shown that the enzyme has a hydrophobic pocket which appears to be less restrictive in orienting the P_1 side chain than that of α -chymotrypsin (Wright, 1972). Moreover, one of the sides of this pocket is more irregular in structure than in α -chymotrypsin and thus may permit more favourable interactions with short aliphatic amino acid residues (Robertus et al., 1972). The sequence data for SGP3 (Johnson and Smillie, 1974) can be interpreted as showing a similar structure of one of the sides of the pocket as in subtilisin. Of course, such features can only be definitely revealed by a detailed X-ray study of SGP3. It appears that such a study, including difference maps of enzyme-substrate complexes, could provide a deep insight into the relationships between primary and secondary specificity.

Our current knowledge of the importance of secondary enzyme-substrate interactions in serine proteases, except for the works of Thompson and Blout (1970; 1973 b-d) and the present papers (IV-VI), stems largely from studies with ester substrates (Mori-hara and Oka, 1973 b; Mori-hara et al., 1974). Although k_{cat} for ester substrates are usually thought to represent k_3 , which has been shown to be little affected by secondary interactions in elastase (Thompson and Blout, 1970), there are strong indications that the majority of the enzymes tested have a rate of hydrolysis markedly dependent on substrate chain length. However, the trypsinlike enzymes, both of mammalian and microbial origin, show very

little dependence on chain length, probably due to very strong primary interactions (Mori-hara and Oka, 1973 b). α -Chymotrypsin, which shows strong primary interactions, appears to be intermediate between the above mentioned two kinds of proteases, showing only moderate dependence on chain length (Mori-hara and Oka, 1973a ; IV). As mentioned in paper IV it appears that the relative order of importance of secondary enzyme-substrate interactions is the inverse of that for primary interactions for a given set of enzymes. It can be expected, then, that enzymes which are highly dependent on secondary enzyme-substrate contacts will show less stringent specificity towards the P_1 amino acid residue than those with a strong primary interaction.

In conclusion it may be worthwhile to mention the following statement from paper IV: Evolution seems to have presented proteolytic enzymes with two potential strategies in catalyzing substrate hydrolysis. The first strategy has evolved enzymes with strong primary specificity, such as the trypsins and α -chymotrypsin. A disadvantage of this type of enzyme is that it is severely restricted in the type of bond it can cleave. The second strategy has evolved enzymes which for their activity, are also highly dependent on enzyme-substrate interactions occurring outside the primary specificity site. Examples of such enzymes are elastase and SGP3. The disadvantage of this strategy is that such enzymes are inefficient catalysts of the hydrolysis of short peptides. Since the efficiency of the catalytic process is subject to limits other than those stemming from the contact between enzyme and substrate, one of these strategies can only be exploited fully at the expense of the other.

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APPENDIX

A key to scientific research literature^{x)}

What was said

What was meant

Introduction

It has long been known that.....

I haven't bothered to look up the original reference but.....
Interesting to me.

Of great theoretical and practical importance.....

While it has not been possible to provide definite answers to these questions.....

The experiment didn't work out, but I figured I could at least get a publication out of it.

Experimental Procedure

The W-PO system was chosen as especially suitable to show the predicted behaviour.....

The fellow in the next lab had some already made up.

Three of the samples were chosen for detailed study.....

The results on the others didn't make sense.

Accidentally strained during mounting.

Dropped on the floor.

Handled with extreme care throughout the experiment.....

Not dropped on the floor.

Results

Typical results are shown.....

The best results are shown.

Agreement with the predicted curve is:

 excellent.....

fair

 good.....

poor

 satisfactory.....

doubtful

 fair.....

imaginary

It is suggested that...It is believed that...It may be that.....

I think

Discussion

It is generally believed that.....

A couple of other fellows think so too.

It is clear that much additional work will be required before a complete understanding.....

I don't understand it.

Unfortunately, a quantitative theory to account for these results has not been formulated.....

Neither does anybody else.

Correct within an order of magnitude..

Wrong.

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Glotz did the work and Doe explained what it meant.

^{x)}From a bulletin board in the University of Georgia Postgraduate School.

